Insect retinal pigments

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CHAPTER 11

Insect Retinal Pigments: Spectral Characteristics and Physiological Functions

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1. INTRODUCTION

The principal pigments in the insect retina are, of course, the visual pigments, anchored in the membrane of the photoreceptor cells. The photoreceptor cells also contain, like almost all eukaryotic cells, numerous mitochondria that harbour a second class of pigments, the mitochondrial cytochromes and

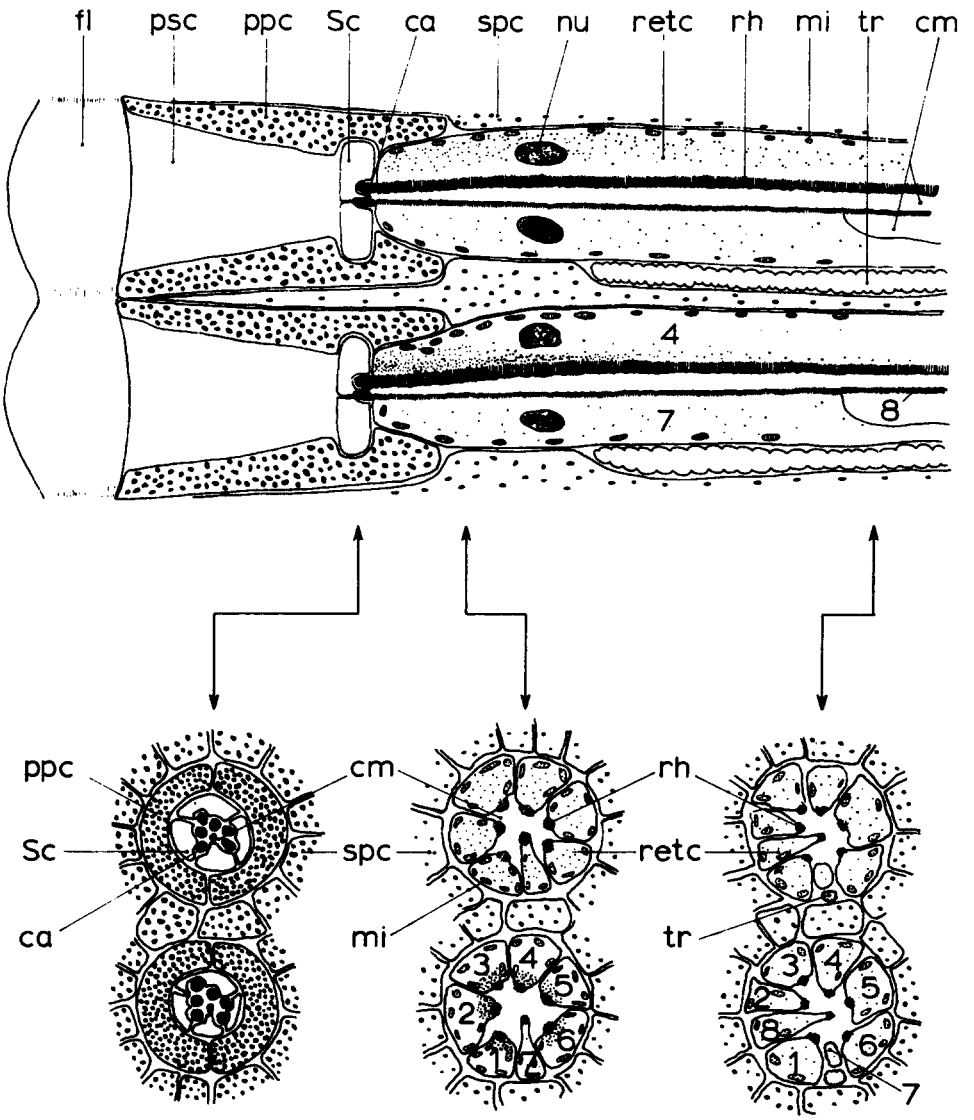


Fig. 1. Two ommatidia of a blowfly eye in the dark-adapted (upper ommatidium) and light-adapted state (lower ommatidium), respectively. A fly ommatidium consists of a facet lens (fl), a pseudocone (psc), four Semper cells (Sc), eight photoreceptor, visual sense or retinula cells (retc), numbered R1 to R8, two primary pigment cells (ppc), six secondary pigment cells (spc) and a trachea (tr). The membrane of the visual sense cells is folded at one side of the cell into microvilli, that together form a rhabdomere (rh); this is the part of the cell membrane that harbours the visual pigment molecules. The rhabdomeres, separated from each other by the central matrix (cm) and distally fitted with a cap (ca), are effective, individual optical waveguides (cross-section 1–2 μm). Whilst the rhabdomeres of the large photoreceptors, R1-6, stretch over the whole length of the retina (ca. 250 μm), those of R7 and R8 share this path: the rhabdomere of R8 is precisely in tandem with that of R7, thus making up one optical waveguide. The photoreceptor cells furthermore contain a nucleus (nu) and numerous mitochondria (mi). In the dark-adapted state, pupillary pigment granules inside the photoreceptor cell are withdrawn from the rhabdomeric area, but upon light-adaptation, the granules migrate towards the rhabdomere so to obstruct there the light propagating along the optical waveguide. The position of the pigment granules in the screening pigment cells is stable, i.e. independent of light/dark adaptation.

flavoproteins, essential for the mitochondrial oxidative metabolism that fuels the visual process. Moreover, insect photoreceptor cells contain highly mobile light-absorbing granules that together act as a light-control or pupil mechanism. Most conspicuous are the ocular screening pigments, deposited in a

dense assembly of granules in the pigment cells, that paint the insect compound eye: reddish in most flies, brown–black in bees, or a mixture of orange and green in certain dragonflies. In addition to determining the eye's appearance, these pigments play a vital supportive role in insect vision.

By definition, pigments absorb light and hence can be studied by spectrophotometrical methods. This provides the key to a most attractive property of insect retinal pigments, namely their potential in non-invasive, optical probing of the visual process. Indeed, in the recent years, optical methods have greatly contributed to progress in retinal and eye research. This chapter reviews our present knowledge of the insect eye with an emphasis on the phototransduction process. The majority of the literature on insect vision concerns the eye of higher Diptera, especially the fruitfly *Drosophila*. Thus, initially, focus will be on the fly eye, the model in insect vision research. Subsequently, whether the concepts derived from the exemplary fly are applicable to other insect eyes will be considered.

2. MORPHOLOGY OF THE FLY RETINA

The building block of compound eyes is the ommatidium. The number of ommatidia depends on the size of the animal; e.g. there are about 700 ommatidia in an eye of the fruitfly *Drosophila melanogaster*, 3000 in the housefly *Musca domestica*, and well over 5000 in the blowfly *Calliphora vicina* (Hardie, 1985). Nevertheless, all fly ommatidia have essentially the same structure (see Fig. 1). Beneath the corneal facet lens, two primary pigment cells surround the transparent pseudocone. Subsequently, eight photoreceptor or visual sense cells, i.e. six large photoreceptor cells, called R1–6, and two slimmer photoreceptors, R7 and R8, are enveloped by six secondary and six tertiary pigment cells (*Drosophila*: Ready, 1989; *Musca*: Boschek, 1971). Finally, four so-called Semper cells form an interface between pseudocone and photoreceptor cells. Thread-like extensions of the Semper cells expand proximally in the retina and are filled with the basal pigment granules (Hardie, 1985). Tracheoles, extensions of large air sacks in the head, penetrate the retina; one tracheole per ommatidium.

3. FLY VISUAL PIGMENT

The main element of a photoreceptor cell is the long, cylindrical rhabdomere, diameter ca. 1.5 μm and average length 83 μm in *Drosophila* and 250 μm in *Calliphora*. A rhabdomere consists of a dense pile of microvilli, i.e. tube-like protrusions of the cell membrane, average length 1 μm and diameter 60 nm (Hardie, 1985). The microvillar membrane is densely packed with protein molecules of which 65% is visual pigment (Schwemer and Henning, 1984). A blowfly rhabdomere has 1.4×10^5 microvilli and a microvillus can contain well over 1000 visual pigment molecules, so a photoreceptor has about 2×10^8 visual pigment molecules (Hamdorf, 1979). The rhabdomere acts as an optical waveguide, similar to the rods and cones of the vertebrate eye. The primary step in vision occurs here when a visual pigment molecule is hit by a light quantum.

3.1. Photochemical Cycle of Fly Rhodopsin

A fly visual pigment molecule consists of a protein, opsin, binding its rather extraordinary chromophore, 3-hydroxy-retinal, via a Schiff base (Vogt and Kirschfeld, 1984). In the rhodopsin state (also called xanthopsin; Vogt, 1989) the chromophore exists in the 11-*cis* configuration. After photon absorption this transforms into the all-*trans* isomer, which is followed by a series of thermal decay steps ending in a thermostable metarhodopsin state (reviews Hamdorf, 1979; Stavenga and Schwemer, 1984). The reverse process, reconversion to rhodopsin, occurs after photon absorption by metarhodopsin.

The rhodopsin and metarhodopsin of the principal cell class, R1–6, of houseflies and blowflies are characterized by a main absorbance band (the α-band) peaking at $\lambda_{max} \approx 490$ nm and at $\lambda_{max} \approx 580$ nm, respectively; the pigment pair accordingly is indicated by R490–M580. In the fruitfly, the peak wavelength of both the rhodopsin and metarhodopsin are slightly hypsochromically shifted: R480–M570; in the hoverfly *Eristalis* this shift is stronger: R460–M550 (Stavenga and Schwemer, 1984).

The photochemical cycle of the visual pigment has been analyzed in extensive detail in the case of the blowfly *Calliphora*. The lifetime of the thermolabile intermediate states has been determined by *in vivo*

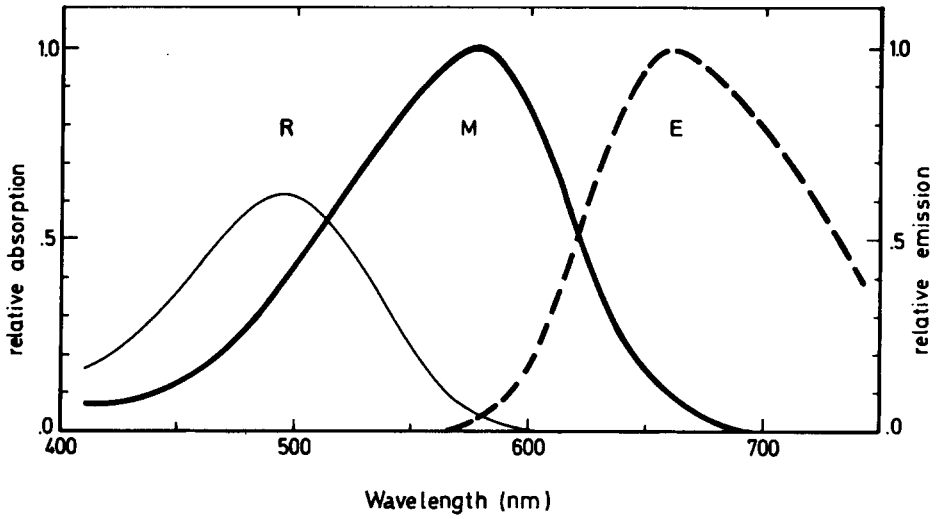


Fig. 2. Absorbance spectra of the rhodopsin (R) and metarhodopsin (M) of blowfly photoreceptor cells R1–6, together with the emission spectrum of the metarhodopsin (E); from Stavenga (1983).

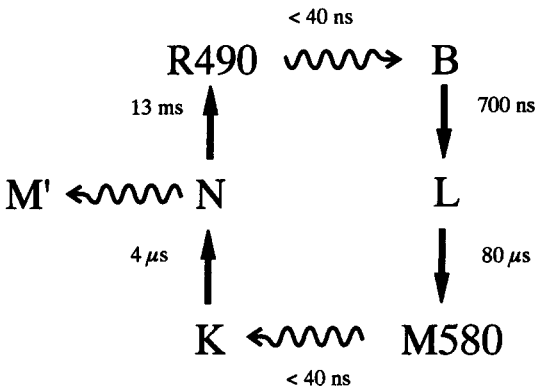


Fig. 3. Photochemical cycle of blowfly visual pigment with thermal decay time constants. Upon photon absorption, rhodopsin (R490) converts via the thermolabile intermediate states bathorhodopsin (B) and lumirhodopsin (L) into the thermostable metarhodopsin (M580). Reconversion of metarhodopsin to rhodopsin occurs via intermediates K and N (Roebroek *et al.*, 1989). At high intensities another photostable metarhodopsin, M', is populated; presumably via photoconversion of N.

laser microspectrophotometry (Kruizinga *et al.*, 1983, see also Roebroek *et al.*, 1989; Fig. 3).

Prolonged illumination with monochromatic light of moderate intensity establishes a photosteady state, or photoequilibrium, where the ratio between the rhodopsin and metarhodopsin population is determined by the ratio of their absorbance coefficients at the wavelength of illumination. Red light establishes a virtually pure rhodopsin population, whilst blue light can result in a rhodopsin low of 30% together with 70% metarhodopsin (Hamdorf, 1979).

A quite distinct difference between the two members of the rhodopsin–metarhodopsin pair is their fluorescence. Invariably, the fluorescence of the rhodopsin state is negligible compared to that of metarhodopsin. The emission band of blowfly metarhodopsin is in the red (Fig. 2, λ_{max}= 660 nm; Kruizinga and Stavenga, 1990) and the fluorescence quantum efficiency is sufficient for usage of the emission as an *in vivo* diagnostic for visual pigment content (Stavenga, 1983, 1989; Hofstee and Stavenga, 1995; Fig. 4). Illumination with very bright blue light populates an as yet ill-characterized, thermostable visual pigment state (Franceschini *et al.*, 1981; Stavenga *et al.*, 1984). Its emission spectrum resembles that of metarhodopsin, M, and hence is called M' (Kruizinga and Stavenga, 1990); presumably this state has a 13-*cis* chromophore and is created by photoconversion of intermediate N (Fig. 3).

3.2. Opsin Synthesis and Visual Pigment Renewal

Visual pigment synthesis requires the presence of retinoids to produce chromophore. After incorporation into the membrane, the visual pigment molecules are subject to the continuous process of membrane

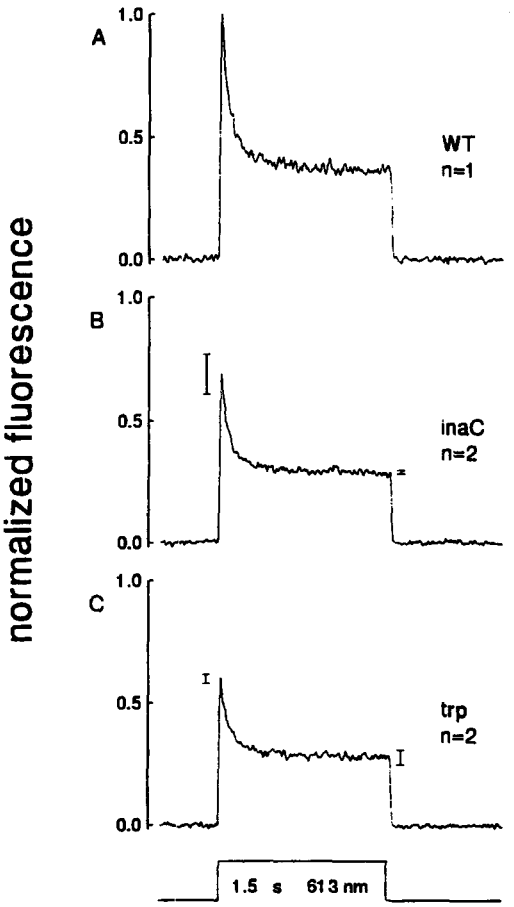


Fig. 4. Measurements of visual pigment content of wild-type (WT, A) *Drosophila* and the phototransduction mutants *inaC* (B) and *trp* (C). The red (613 nm) induced emission (> 670 nm) was measured from the deep pseudopupil in the eye of intact, living flies. The eyes were first irradiated with intense blue (456 nm) light, to establish a maximal metarhodopsin content, and then > 1 min dark adapted. The fluorescence measurements were normalized with respect to the initial, peak value of wild-type. The peak to background difference was calculated and the values of *inaC* and *trp* were divided by the corresponding value of wild-type. It followed that both the visual pigment content of the tested *inaC* and *trp* mutants appeared to be reduced, i.e. to 64 ± 13% and 51 ± 3%, respectively, of that in wild-type (Hofstee and Stavenga, in prep.)

breakdown and renewal. More important, fly visual pigment is vulnerable to direct and specific enzymatic attack as demonstrated by Schwemer (1989, 1993) in his extensive analyses of the blowfly *Calliphora*, carried out by a combination of spectrophotometrical methods and HPLC. Crucial in the enzymatic degradration process is the state of the visual pigment: whereas rhodopsin R490 is rather stable in darkness—it declines slowly, with a halftime of about 130 hr—metarhodopsin M580 decays 60 times faster and hence is selectively degraded (Schwemer, 1989). With 700 metarhodopsin molecules per microvillus, decaying in a 130 min halftime process, at most, six metarhodopsin molecules are degraded per min in one microvillus, i.e. only one metarhodopsin molecule per 10 s.

The opsin is broken down intracellularly and the chromophore, all-*trans*-3-hydroxy-retinal, after its release, is reisomerized to 11-*cis*-3-hydroxy-retinal and subsequently rejoined with newly built opsin through a complex, yet efficient pathway (Fig. 5, Schwemer, 1993). Firstly, the liberated chromophore is transported from the visual sense cell to the primary pigment cell where it is bound by a light-driven isomerase. The created complex absorbs in the violet ($\lambda_{max} \approx 410$ nm), so that illumination induces isomerization of the bound chromophore from the all-*trans* to the 11-*cis* configuration, followed by release of the 11-*cis*-3-hydoxyretinal. The chromophore thus is ready for reuse, but first it has to be redirected to the visual sense cells. Probably the chromophore is temporarily reduced to the alcohol and then transported back again by a retinal-binding protein. Subsequently a new, functional rhodopsin molecule is synthesized in the photoreceptor cell, which then is inserted into the rhabdomeric membrane (Schwemer, 1993; Fig. 5).

Most probably, visual pigment renewal in other flies occurs through an identical route (for the case of *Drosophila*, see Isono *et al.*, 1988, and Ozaki *et al.*, 1993). The general rule seems to be that, quite practically, the fly makes good use of light energy that enters the ommatidia from oblique, off-axis directions. Most of this light is absorbed in the primary pigment cells by the screening pigment and hence is wasted. At least a small part of the incident light is absorbed there by the photoisomerase and thus can serve a higher goal by isomerizing the degraded chromophore and preparing it for another round in the phototransduction process.

The isomerase only absorbs in the violet. Hence no renewal will occur under green illumination, however, green light does create a substantial metarhodopsin fraction, which is selectively degraded (see above). This leads to a decline of the visual pigment concentration in the visual membrane, yielding a

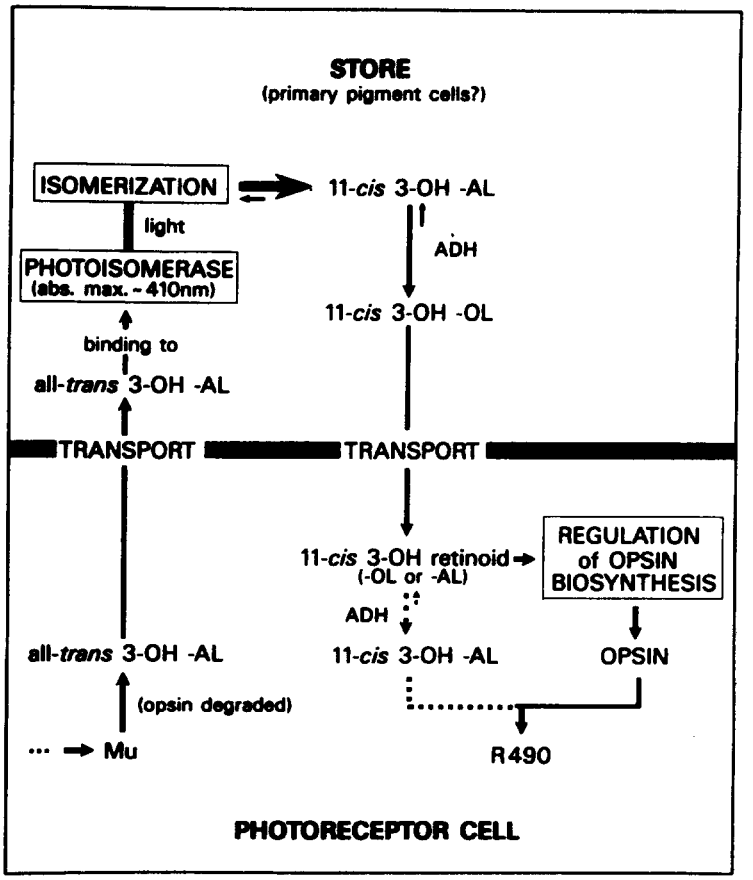


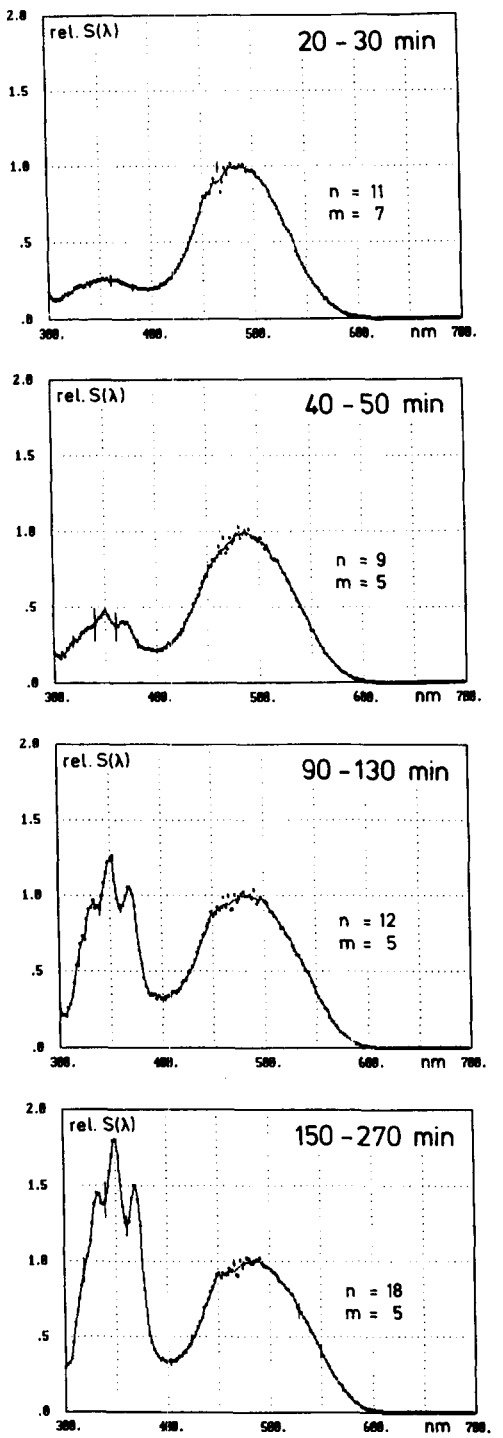
Fig. 5. Cycle of the visual pigment chromophore of blowfly rhodopsin. After degradation of metarhodopsin, all-*trans*-3-hydroxyretinal is released and transported to the primary pigment cells. There it is bound by the photoisomerase and, upon photon absorption, converted to 11-*cis*-3-hydroxy-retinal. After reduction, the chromophore is re-routed to the photoreceptor cells and then, after being oxidized to the aldehyde, combined with newly synthesized opsin (from Schwemer, 1993).

gradually decreasing visual sensitivity. This process is only reversed when 11-*cis*-3-hydroxy-retinal is supplied, either exogenously and/or endogenously via illumination with violet/violet light (Schwemer, 1989; Hamdorf *et al.*, 1992; Fig. 6).

Flies reared on a vitamin A-deprived diet have a low visual sensitivity, clearly due to the inability to produce visual pigment (Goldsmith *et al.*, 1964). In those flies, opsin mRNA and opsin peptide exists in an intermediate step of post-translational processing. Carotenoid replacement promotes the recovery with an increase of the chromophore, as well as the opsin. Indeed, supplying the chromophore, 11-*cis*-3-hydroxy-retinal, to carotenoid-deprived *Drosophila* and *Calliphora* induces maturation of the opsin, presumably because the chromophore post-translationally controls expression of the apoprotein (Isono *et al.*, 1988; Schwemer and Spengler, 1992; Ozaki *et al.*, 1993).

3.3. Sensitizing Pigment

Flies reared on a vitamin A-deprived diet not only have a low visual sensitivity; the sensitivity in the UV relative to the peak in the blue–green is then much depressed (Stark *et al.*, 1977). The reason is that fly visual pigments, in addition to the chromophore 3-hydroxyretinal, bind 3-hydroxy-retinol (Fig. 7; Vogt, 1989). When this ultraviolet-absorbing compound is excited by a photon, it transfers the absorbed energy to the chromophore which then isomerizes as usual (Kirschfeld *et al.*, 1977). The 3-hydroxy-retinol



therefore acts as a sensitizing or antenna pigment (Kirschfeld, 1986). Energy transfer can occur from the excited sensitizing pigment to both rhodopsin and metarhodopsin (Minke and Kirschfeld, 1979). The sensitization of rhodopsin by the ultraviolet-absorbing antenna pigment is the reason for the strongly enhanced spectral sensitivity in the ultraviolet, measured in physiological responses (Hardie, 1986).

Hamdorf *et al.* (1992) have investigated this observation in a detailed *in vivo* electrophysiological study on the speed of incorporation of various retinoids in the visual pigment molecules. The retinoids (e.g. retinal or retinol in either the all-*trans* or the 13-*cis* configuration) were applied with a most simple 'perfusion' technique, also applied by Rubinstein *et al.* (1989) in a study of chemically-induced retinal degeneration in the *Drosophila rdgB* mutant (Section 6). A drop containing dissolved retinoid was administered to the cornea of an immobilized blowfly *Calliphora*, white-eyed mutant *chalky*. The fly started with a very low visual pigment content as it was reared on a vitamin A-deprived diet. The spectral sensitivity then was measured at various times after application of the retinoid. As an example, Fig. 6 presents spectral measurements after application of all-*trans*-retinal to the cornea, normalized to the sensitivity peak in the visible. Initially, the sensitivity band in the UV is very low and smooth. Within a few hours, this band has risen considerably and then features a prominent vibronic fine structure, with peaks at 333, 350 and 369 nm. These peaks prove the presence of 3-hydroxy-retinol. Evidently, this derivative was enzymatically produced from the administered all-*trans*-retinal. Calculations of energy transfer indicate that in retinoid-saturated eyes at least two sensitizing pigment molecules can be bound to one visual pigment molecule (Hamdorf *et al.*, 1992). Supposing that 1000 molecules per microvillus ultimately are supplied with two sensitizers in

Fig. 6. Sensitivity spectra, S(λ), of blowfly photoreceptors determined from intracellular recordings after application of all-*trans*-retinal to the cornea. Initially, the fly has a low visual pigment due to rearing on a vitamin A-deprived diet. The spectral sensitivity in the ultraviolet then is low, but uptake of the retinoid results in visual pigment synthesis and progressively binding of the sensitizing, antenna pigment 3-hydroxy-retinol by the visual pigment molecules. This is expressed by the enhanced sensitivity in the ultraviolet and the appearance of a fine structure in the UV sensitivity band (from Hamdorf *et al.*, 1992).

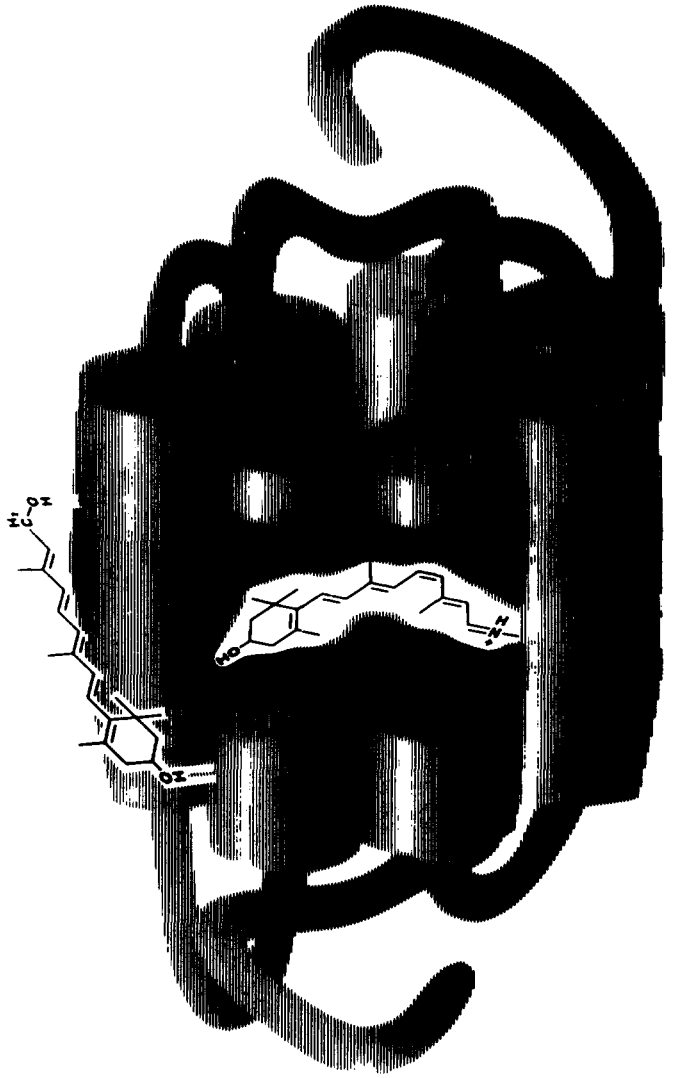


Fig. 7. Model of fly rhodopsin with chromophore 11-*cis*-3-hydroxy-retinal (central), covalently bound to the 7-helical opsin through a Schiff-base together with one molecule of sensitizing pigment, 3-hydroxy-retinol, presumably bound by hydrogen bonds (from Vogt, 1989).

a process with halftime 100 min (Hamdorf *et al.*, 1992), at most once per 3 s a sensitizing molecule is bound to a visual pigment molecule in each microvillus.

Carotenes, like β-carotene, lutein or zeaxanthin cannot serve as precursors for the sensitizing pigment if applied to the cornea. Interestingly, vitamin A2, 3-dehydro-retinol, is taken up and can function as a sensitizing pigment as followed from the slightly different vibronic structure of the UV-band: the 350 and 369 nm peaks are shifted to 358 and 380 nm, respectively. However, after a few hours, the normal UV fine structure emerges, indicating replacement of the 3-dehydro-retinol by 3-hydroxy-retinol (Hamdorf *et al.*, 1992).

4. PHOTOVOLTAGES AND CURRENTS

Light absorption by visual pigment causes electrical changes that are measurable in the intact eye by intracellular recordings as well as in the

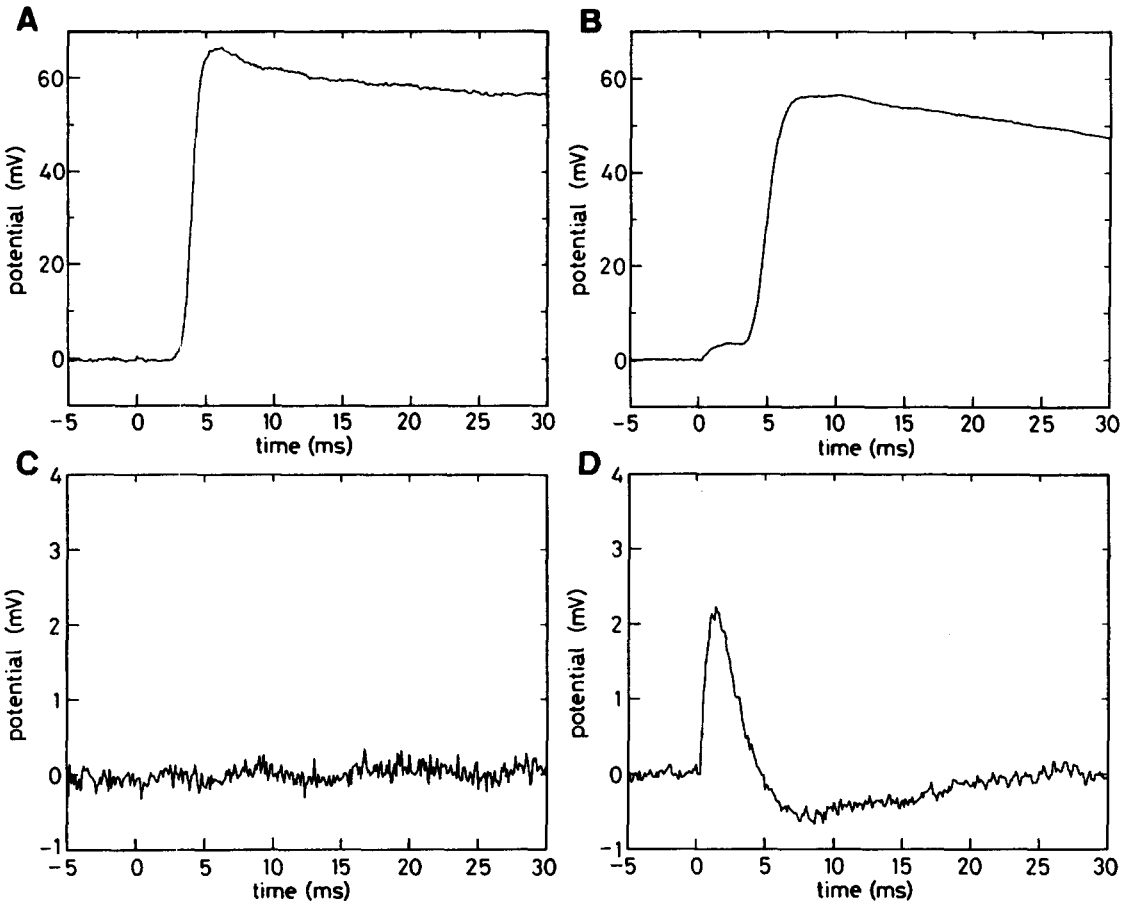


Fig. 8. Early and late receptor potential recorded from blowfly photoreceptor cells. At t = 0, an intense blue flash was delivered to a red-adapted eye, i.e. when virtually all the visual pigment molecules are in the rhodopsin state (A, C); or a red flash was given to a blue-adapted eye, i.e. with 70% of the visual pigment molecules in the metarhodopsin state (B, D). The animal was in normal air in A and B, and in a nitrogen atmosphere in C and D. Under anoxia only an early receptor potential (ERP) can be evoked. A biphasic ERP occurs upon conversion of metarhodopsin (D) due to the slow conversion of intermediate N into R (see Fig. 3). Rhodopsin conversion yields a silent ERP due to the rapidity of the forward conversion process (C). The late receptor potential (LRP) induced by the blue flash (A) is extreme due to the excessive creation of metarhodopsin; the receptor remains depolarized during a prolonged period, i.e. several tens of seconds (a PDA). The late receptor potential induced by the red flash is terminated much more rapidly (not shown), because only a limited number of metarhodopsin molecules is created (B). Superimposed on the LRP, the first phase of the ERP is prominently visible (from Gagné *et al.*, 1989).

electroretinogram (ERG; see Hamdorf, 1979; Minke and Kirschfeld, 1980). In recent years, an additional, most powerful tool has been developed, namely patch-clamp of isolated photoreceptor cells (Ranganathan *et al.*, 1991; Hardie and Minke, 1992). In combination, the electrical approaches have been instrumental in delineating the primary visual process.

4.1. Early Receptor Potential and Current

The light-induced transformations in a visual pigment molecule are accompanied by charge displacements, i.e. by currents. Since visual pigment molecules are integral membrane proteins, this current is measurable when the net direction is perpendicular to the membrane as the current then changes the charge of the membrane capacitor. This creates a change in membrane potential, called the early receptor potential (ERP).

Figure 8 presents intracellular recordings from a blowfly *Calliphora* photoreceptor, illuminated with a 1 ms flash, sufficiently intense to establish a

photoequilibrium. Conversion of fly rhodopsin (R) to metarhodopsin (M) yields a negligible ERP (Fig. 8C). The reverse conversion induces a distinct biphasic potential change (Fig. 8D). As indicated in Fig. 3, R to M conversion is complete within ca. 0.1 ms, but M to R conversion proceeds much slower, through an intermediate that thermally decays with a time constant of 13 ms (Roebroek *et al.*, 1989). Because a typical blowfly photoreceptor has a membrane resistance in the dark-adapted state of 25–35 MΩ (Van Hateren, 1986) and has a membrane surface of 1.7×10^4 μm^2 (Hardie *et al.*, 1981), which yields a membrane capacity of 170 pF when taking the usual 1μF·cm^{-2}, its membrane time constant is about 5 ms. Hence, R to M conversion is complete within the membrane RC-time, but M to R conversion is too slow and hence a distinct ERP can be measured. Gagné *et al.* (1989) calculate that M to N conversion induces the movement of effectively 0.03 electron charge accross the membrane and that N to R conversion moves 0.04 electron charge in the opposite direction. Evidently, although the charge displacement across the membrane thickness occurring in a single molecule is in fact minute, a quite well measurable ERP occurs when a large fraction of the 2×10^8 visual pigment molecules per cell are rapidly converted.

The second phase of the fly ERP normally drowns in the voltage changes resulting from the phototransduction process (Fig. 8B). Blocking the latter by anoxia (Fig. 8C and D), however, or by some photoreceptor mutation, as in the *Drosophila norpA* (*no receptor potential A*) mutants (Pak and Lidington, 1974), yields full exposure of the ERP.

The ERP is a direct result of intramolecular charge movements and thus, in principle, could provide insight in the process of light-triggered molecular reorganizations. Although the ERP probably is too coarse a tool for such a goal, it is at least demonstrably quite valuable in assessing the rhodopsin concentration in individual visual sense cells (Pak and Lidington, 1974; Minke and Kirschfeld, 1980).

The early receptor current (ERC), the cause of the ERP, can be most exquisitely measured on dissociated photoreceptors with the patch-clamp technique. Whole cell recordings from *Drosophila* photoreceptors yield a biphasic ERC, as the ERP, but the effect of the strongly limiting RC-filter of the cell membrane now is elegantly avoided (Hardie, 1995).

4.2. Light-induced Current and Late Receptor Potential

The rhabdomere contains, in addition to the bulk protein rhodopsin, a whole menagery of molecules that together make up the machinery of the phototransduction process. Phototransduction in insects is the subject of intense research of several groups, using mainly *Drosophila* as the experimental animal. The great potential of the fruitfly is based on the continuously increasing number of visual mutants. Its accessibility for genetic engineering is described in several recent reviews (Ranganathan *et al.*, 1991; Smith *et al.*, 1991; Minke and Selinger, 1992; Selinger *et al.*, 1993; Hardie and Minke, 1993; Wu *et al.*, 1995).

Fly phototransduction belongs to the family of G-protein-coupled second-messenger systems. A visual pigment molecule, after photoconversion to its metarhodopsin state, couples to a G-protein, G_q, and then is able to catalyze GDP–GTP transfer. G_q·GTP activates phospholipase C (PLC), that in turn hydrolyzes phosphatidylinositol bisphosphate (PIP_2) into inositoltrisphosphate (IP_3) and diacylglycerol (DAG). The released IP_3 triggers efflux of calcium from the submicrovillar cisternae (SMC; Fig. 9), a calcium store bordering the rhabdomere. The DAG, together with Ca^{2+}, is assumed to activate photoreceptor protein kinase C (PKC), which has a regulatory role on intracellular calcium (Fig. 9). The end effect is the opening of ion channels in the cell membrane, resulting in ion currents (light-induced current, LIC; Hardie, 1991a) and depolarization of the cell membrane (late receptor potential, LRP; Hochstein *et al.*, 1973). The last steps of the mainstream phototransduction cascade are not yet fully resolved, however, and many regulatory side-loops remain to be elucidated and/or await discovery. There is nevertheless accumulating evidence that the light-sensitive channels are located at the base of the microvilli, in the cell membrane near the submicrovillar cisternae (Fig. 9) and that the microvillus can be regarded as a functional unit in phototransduction (Hamdorf, 1979; Hardie and Minke, 1992, 1993; Pollock *et al.*, 1995). According to Minke and Selinger (1992) there are 40 rhodopsin molecules per G-protein, i.e. each microvillus contains no more than 25 G-protein molecules.

The influx of calcium into the photoreceptor cell is an important component of the LIC. Using

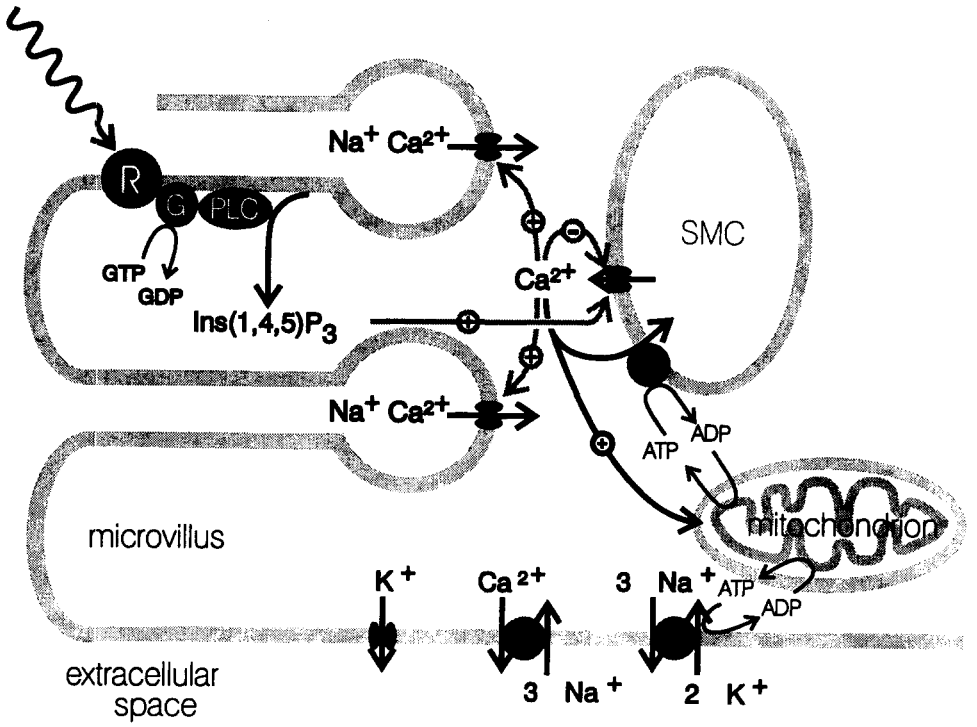


Fig. 9. Diagram of the phototransduction machinery of a fly photoreceptor and its coupling with mitochondrial activity. Photoactivated rhodopsin (R), i.e. metarhodopsin, activates a G-protein (G), causing GDP–GTP transfer; the thus-activated G-protein activates phospholipase C (PLC) which hydrolyzes PIP_2 into DAG and IP_3. The IP_3 activates calcium channels in the submicrovillar cisternae (SMC). The released calcium plays a key role in opening plasma membrane channels as well as in activating the mitochondrial respiratory chain. This recycles the ATP necessary for the sodium pump that maintains the sodium and potassium gradients accross the cell membrane and the calcium pump in the SMC membrane. The cell membrane, furthermore, contains voltage-dependent potassium channels and sodium–calcium exchanger molecules (modified from Mojet, 1992).

isolated photoreceptors loaded with fluorescent calcium indicators, Peretz *et al.* (1994a, b) and Ranganathan *et al.* (1994) obtained direct proof of a substantial light-induced increase of the intracellular calcium concentration.

4.3. Prolonged Depolarizing Afterpotential

Deactivation is an essential part of any signal transducing mechanism. In other words, the capacity of metarhodopsin to trigger the phototransduction process has to be short-lived. Metarhodopsin deactivation is achieved via phosphorylation by rhodopsin kinase (Fig. 10; Bentrop and Paulsen, 1986; Doza *et al.*, 1992; Byk *et al.*, 1993) and binding of two types of arrestin molecules (Dolph *et al.*, 1993); the arrestins are also called phosrestins, because they are rapidly phosphorylated after light onset (Matsumoto and Yamada, 1991; Matsumoto *et al.*, 1994).

The limited amount of rhodopsin kinases and arrestins in a photoreceptor cell is sufficient to rapidly inactivate the newly created metarhodopsin molecules under a light regime where the production of metarhodopsin molecules is low. However, in a procedure where the production of metarhodopsin molecules is excessive, as occurs with a bright, blue flash converting a large number of rhodopsin molecules (as occurred in the experiment of Fig. 8A), it can take a considerable time after the flash before all metarhodopsins are knocked out of action. Consequently, during that time, the light-induced membrane depolarization continues in the dark: the prolonged depolarizing afterpotential (PDA); see Minke (1986).

A proviso for induction of a PDA is, of course, that the photoreceptor must have a high visual pigment content. In fact, the excitability of the PDA appears to be crucially dependent on the rhodopsin concen-

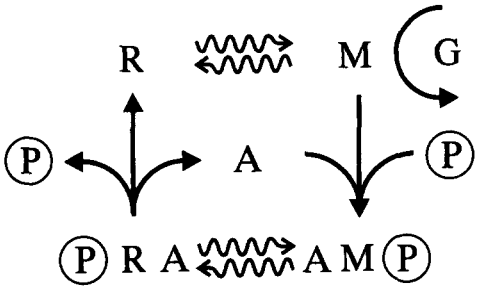


Fig. 10. Simplified diagram of the deactivation and regeneration of fly visual pigment. Newly created metarhodopsin (M) can interact with a G-protein (G) until it is deactivated by phosphorylation (by rhodopsin kinase), and by binding arrestin (A). The photointerconvertibility of rhodopsin and metarhodopsin is presumably rather independent of phosphorylation and presence of arrestin. Arrestin is thermally released from photoregenerated rhodopsin and this is dephosphorylated by rhodopsin phosphatase. The freed rhodopsin then is ready for renewed participation in the phototransduction process.

tration, as was demonstrated specifically by Pak (1979), who exploited the PDA as a most useful diagnostic in screening fruitflies by ERG.

In a normal *Drosophila* photoreceptor cell, a PDA is initiated when more than ca. 20% of the visual pigment molecules is converted to the metarhodopsin state (Stephenson and Pak, 1980; Minke, 1986). This amount is approximately equivalent to the total number of arrestin molecules in a photoreceptor (Matsumoto and Yamada, 1991; Matsumoto *et al.*, 1994), i.e. there are about 200 arrestin molecules per microvillus in *Drosophila*. In fact, Dolph *et al.* (1993) reported that in mutants lacking arrestin, a PDA is already induced by conversion of less than 0.2% of the rhodopsin to metarhodopsin; i.e. 1 metarhodopsin molecule per microvillus then appears to be sufficient for inducing a PDA.

Whereas in *Drosophila* a PDA lasting several hours can be induced, in the blowfly *Calliphora* (and the housefly *Musca*) a PDA lasting longer than about 1 min is rare, even with the extreme fraction of about 70% metarhodopsin and having the rhabdomeric membrane fully loaded with visual pigment molecules. In photoreceptors of the blowfly, a pronounced PDA can be only produced when the eye is preadapted with intense red light (thus establishing a photochemical steady state with a high concentration of rhodopsin), is allowed a subsequent dark adaptation time of > 1 min (sufficient to dephosphorylate the rhodopsin molecules), and then is delivered a brief, intense blue light (thus creating a huge number of fresh metarhodopsin molecules). In other words, induction of a PDA in a blowfly photoreceptor requires that the unphosphorylated rhodopsins are massively converted. It may seem therefore that arrestin binding and phosphorylation of metarhodopsin do not proceed identically in the different fly species. However, it can be calculated from Bentrop *et al.* (1993) that blowfly photoreceptor cells have just about sufficient arrestin molecules to neutralize all metarhodopsins that are created by (quite unphysiological) pure blue light.

Quite logically, a PDA can be undone by reducing the metarhodopsin concentration present. This occurs when the metarhodopsin is enzymatically degraded. However, this process is slow: in *Drosophila* with an estimated time constant of the order of 6 hr (Pak and Lidington, 1974; compare the ca. 2 hr of *Calliphora*, Section 3.2). A much better alternative therefore is to photoregenerate the metarhodopsin.

5. PHOTOREGENERATION

Deactivated metarhodopsin molecules can no longer activate the G-protein (Selinger *et al.*, 1993), but they are revived by photoregeneration (Fig. 10). This process starts with photoreconversion of metarhodopsin to rhodopsin. Of course, initially this state is phosphorylated and has bound arrestin (Dolph *et al.*, 1993). The latter is rapidly released, however, and dephosphorylation by rhodopsin phosphatase occurs (Byk *et al.*, 1993). Phosphatase activity appears to be highly dependent on Ca^{2+} (Byk *et al.*, 1993) and hence on calcium removal (Selinger *et al.*, 1993). Only when the rhodopsin molecules are fully dephosphorylated are they ready to participate again in the phototransduction process.

Photoregeneration is a direct result of the thermostability of metarhodopsin. It occurs continuously in the natural, physiological situation. Illumination of a fly photoreceptor with broad-band white light yields a photosteady state with about 30% of the visual pigment molecules in the metarhodopsin state (Stavenga *et al.*, 1973). The population is not homogenous throughout the rhabdomere, as the spectral distribution of the propagating light changes due to filtering by the visual pigment itself. The prime, well-

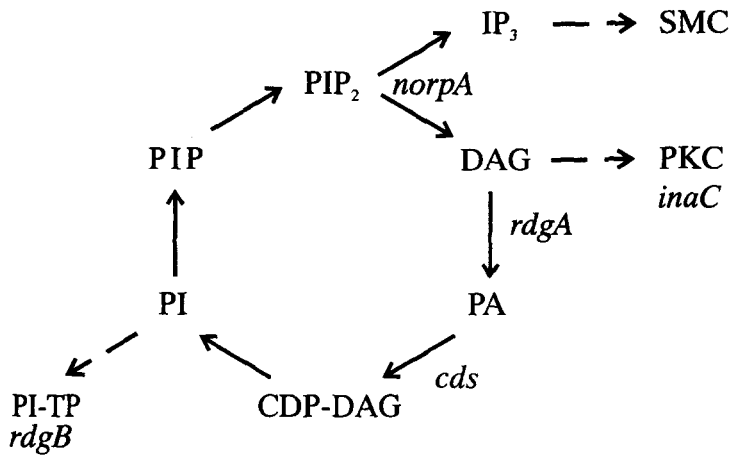


Fig. 11. Diagram of the phosphatidylinositol-4,5-bisphosphate (PIP_2) cycle in *Drosophila* photoreceptor cells. PLC, encoded by the *norpA* gene, splits PIP_2 in IP_3 and DAG. A photoreceptor-specific DAG kinase, encoded by the *rdgA* gene, a CDP–DAG synthase, encoded by the *cds* gene, and a phosphatidylinositol transfer protein, encoded by the *rdgB* gene, participate in PIP_2 acivation and resynthesis (after Wu *et al.*, 1995).

known effect of this so-called self-screening is the broadening of the spectral sensitivity curve with respect to the rhodopsin absorbance curve, but it should be noticed that self-screening also results in more and more yellow light in the proximal part of the rhabdomere. With incident white light, the metarhodopsin fraction will hence gradually decline along the rhabdomere length.

Under natural daylight never more than approximately 5–10% of the visual pigment molecules exist in the metarhodopsin state owing to the screening pigment that acts as a red filter (see Section 9). Therefore, under normal conditions, the number of arrestin molecules is always more than sufficient to quickly deactivate photoconverted visual pigment molecules, so preventing a PDA and its concurrent, disastrous visual desensitization.

6. PHOTORECEPTOR MUTANTS

Response inactivation and the PDA phenomenon are expressions of the phototransduction process under rather extreme treatments. Nevertheless, they appear to be sensitive tests in the discrimination of visual mutants. Pak and co-workers used the degree of desensitization of the light response and the possibility to evoke a PDA to discriminate various classes of *Drosophila* visual mutants (Pak, 1979; Smith *et al.*, 1991; Ranganathan *et al.*, 1991). The isolation of the *ninaE* (*n*either *i*nactivation *n*or *a*fterpotential *E*) mutant may be heralded as a major breakthrough in the history of invertebrate visual pigment research. In *ninaE* mutants, the defective gene results in a low amount of visual pigment resulting in a depressed PDA (Scavarda *et al.*, 1983). Cloning of the *ninaE* gene demonstrated that its product is the opsin in R1–6 photoreceptor cells (O'Tousa *et al.*, 1985).

The visual mutants *norpA*, *inaC* and *trp*, with defects in the PLC, PKC and a calcium channel (the so-called TRP channel), respectively (Bloomquist *et al.*, 1988; Smith *et al.*, 1991; Hardie and Minke, 1992), have been instrumental for our present insight in fly phototransduction. A severe *norpA* (*no r*eceptor *p*otential *A*) photoreceptor cell remains electrically silent, and a dark-adapted *trp* mutant features only a *t*ransient *r*eceptor *p*otential when illuminated with a bright step of light. The magnitude and time course of the light-induced calcium changes appears to depend critically on these mutations (Peretz *et al.*, 1994a, b). In a severe *norpA* mutant, intracellular calcium remains low, which is intelligible when phototransduction is fully blocked by the defective PLC. Furthermore, in the *trp*, only a small and transient calcium increase can be induced. However, in the *inaC* (response *i*nactivation but *n*o *a*fterpotential *C*) mutant, the duration of the receptor

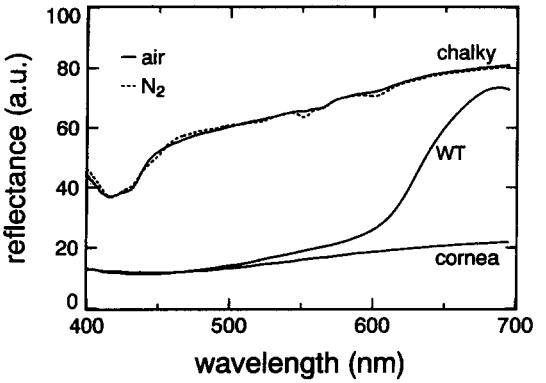


Fig. 12. Reflectance spectra in air (solid curves) or nitrogen (dashed curve) from eyes of intact white-eyed mutant (*chalky*) and wild-type (WT) blowfly *Calliphora vicina*. The two spectra are virtually identical for the wild-type. Also the reflectance spectrum for an isolated, cleaned cornea is given. This is a background signal in both mutant and wild-type flies (from Smits *et al.*, 1995).

potential is greatly lengthened compared to that in wild type (Ranganathan *et al.*, 1991; Hardie *et al.*, 1993), and the light-induced calcium influx is much larger than that in a wild-type photoreceptor (Peretz *et al.*, 1994b), clearly indicating that PKC serves to curtail the light-induced increase in cellular calcium.

The visual defects of many more isolated photoreceptor mutants have been identified. For instance, the *ninaC* gene encodes two retinal specific proteins consisting of a protein kinase domain joined to a domain homologous to the head region of the myosin heavy chain (Pak, 1979; Montell and Rubin, 1988; Porter and Montell, 1993; Porter *et al.*, 1993). A further class of important mutants is that of the *r*etinal *deg*eneration mutants. The *rdgC* mutant has a rhodopsin phosphatase deficiency and hence is unable to regenerate phosphorylated rhodopsin into viable, phototransducing rhodopsin (Steele *et al.*, 1992; Byk *et al.*, 1993; Selinger *et al.*, 1993). Presumably, retinal degeneration in the *rdgC* mutant occurs because the maintained state of phosphorylation leaves the rhodopsin vulnerable to excessive enzymatic degradation which is insufficiently compensated by visual pigment renewal (see Section 3.2).

A recent report by Wu *et al.* (1995) reveals that a photoreceptor-specific CDP–DAG synthase, encoded by the *cds* gene, participates in the crucial cycle of phosphatidylinositol-4,5-bisphosphate activation and resynthesis; together with a DAG kinase, encoded by the *rdgA* gene (Masai *et al.*, 1992), a phosphatidylinositol transfer protein, encoded by the *rdgB* gene (Vihtelic *et al.*, 1993) and the *norpA* product PLC, of course (Fig. 11). The PLC is activated by the *Drosophila* $G_{q\alpha}$ protein (DG_q), which is encoded by the *dgq* gene (Lee *et al.*, 1994).

The extensive work on *Drosophila* mutants is gradually revealing intimacies of a signal transduction process that is almost universal among biological cells. The studies demonstrate the value of photoreceptor mutants, not only for understanding vision, but also for cell research in general.

7. MITOCHONDRIAL PIGMENTS AND OXIDATIVE METABOLISM

7.1. Cytochromes and Electron Transfer Chain

Fly photoreceptors contain, as other visual sense cells, numerous mitochondria (Fig. 1). They supply the ATP necessary to fuel the molecular machinery of the photoreceptor. Mitochondria possess a number of cytochromes: pigments, that play a crucial role in cellular respiration, as they participate in a chain of proteins that pass electrons on to oxygen. Of great experimental value is the fact that a cytochrome molecule changes colour when transferring an electron, i.e. when changing from the reduced state to the oxidized state. The absorbance spectra of the two redox states differ characteristically and this allows the study of mitochondrial activity with optical methods, i.e. by absorbance spectroscopy. Classically, this is done on suspensions of mitochondria or isolated cells (Chance and Williams, 1956).

Fly eyes possess an outstanding advantage because of the existence of white-eyed mutants, i.e. mutants that contain no screening pigments. Mitochondrial activity then can be studied via absorbance difference measurements on the eyes of intact, living animals (Stavenga, 1989). Recently, this approach was extended by applying reflectance difference spectroscopy (Smits *et al.*, 1995). Figure 12 presents reflectance spectra from the blowfly *Calliphora vicina* mutant *chalky* measured in air and nitrogen, respectively. The spectrum differs considerably from that of the red-pigmented eye of the wild-type, because the red screening pigment absorbs strongly in the visible

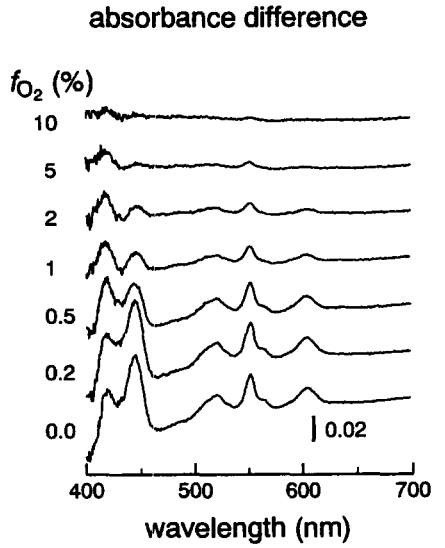


Fig. 13. Absorbance difference spectra calculated from reflectance spectra measured from the dark-adapted white-eyed blowfly *chalky* in air and under hypoxia, indicated by the oxygen fraction f_{O2}. The reflectance spectrum obtained in air served as the reference. Characteristic features are the absorbance increase due to reduction of cyt a (445 and 608 nm), cyt b (564 nm) and cyt c (417 and 552 nm) and an absorbance decrease around 470 nm caused by reduction of cytochromes b and c (from Smits *et al.*, 1995).

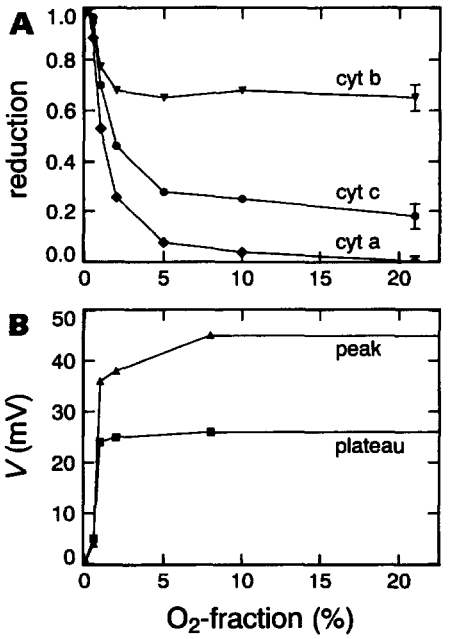


Fig. 14. Dependence on oxygen fraction of peak and plateau values of the light-induced photoreceptor depolarizations (B) and the degree of reduction of cytochromes (A) (from Smits *et al.*, 1995).

wavelength range (Langer, 1975; as shown in Fig. 12, in both wild-type and white-eyes, part of the reflectance originates from the corneal facet lenses). The reflectance spectrum of the white eye has a unique feature, namely a distinct valley in the 410–450 nm range, i.e. where the absorbance by cytochromes is extreme (Tzagaloff, 1982). Anoxia appears to cause a characteristic change in reflectance from the white eye, mainly due to the induced reduction of the mitochondrial cytochromes; in the wild-type eye, the red pigment screen fully obscures this effect. A detailed analysis shows that the spectral changes are extreme at 445 and 608 nm, fingerprinting cyt a (including a_3), at 564 nm, typical for cyt b, and at 417 and 552 nm, characteristic for cyt c (including c_1).

The degree of cytochrome reduction depends on the oxygen pressure as can be assessed from the reflectance difference spectra (Fig. 13). The cytochrome reduction can be compared with the peak and plateau values of the late receptor potential at various values of the oxygen supply (Fig. 14). When a moderately intense light stimulus is applied, peak and plateau height are a measure for light sensitivity, and it thus appears, perhaps surprisingly, that both mitochondrial activity and photoreceptor sensitivity remain virtually unhampered when the oxygen concentration is lowered from the normal 21% down to a few per cent. The fly photoreceptor cell is clearly well-buffered against a low oxygen supply. Oxygen supply is mediated by the retinal tracheoles (Fig. 1). Indeed, the extensive mesh of tracheoles suggests that oxygen consumption by the fly retina is high. Apparently, the design of the retina with its photoreceptor cells is such that visual sensitivity remains robust even at high rates of oxygen consumption and when oxygen supply may be limited.

The conclusion that oxygen consumption is high is in line with the observation that complete oxygen deprivation quickly results in desensitization. Cutting-off the oxygen supply during light inactivates fly photoreceptors in seconds, and even in complete darkness, light sensitivity vanishes within a few minutes (e.g. Smits *et al.*, 1995), indicating that also then oxygen consumption, or, ATP turnover, is far from negligible. The main ATP consumer is the sodium pump in the plasma membrane, which evid-

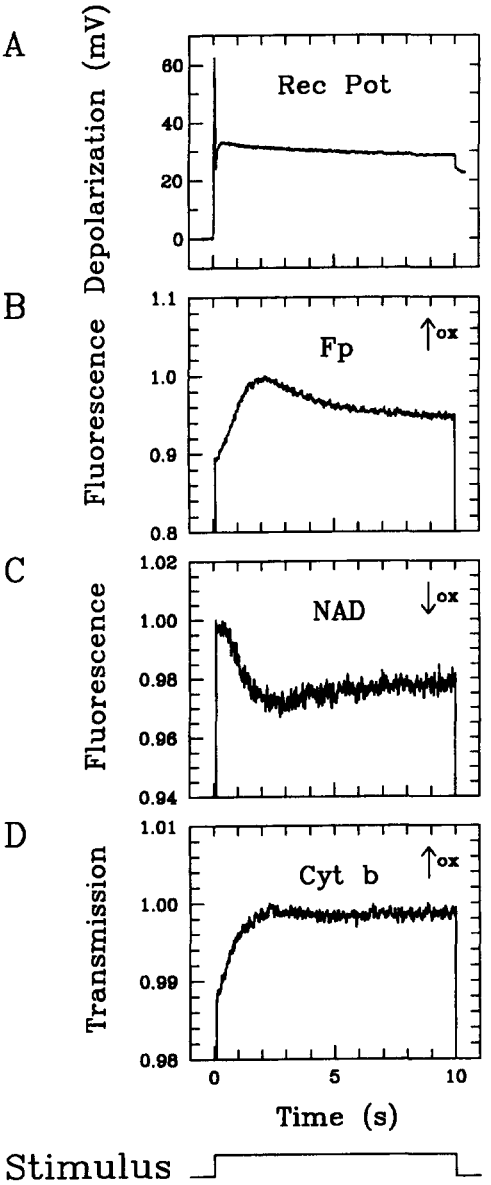


Fig. 15. Bright light-induced (late) receptor potential (A) and accompanying optical changes measured from the eye of a white-eyed blowfly, *chalky*. The blue-induced green fluorescence (B) exhibits a biphasic emission increase, indicating an increase in oxidized flavoprotein, Fp. The UV-induced blue fluorescence (C) exhibits an emission decrease due to an increase in oxidized NADH. The increase in blue (433 nm) transmission (D) reflects an increase in oxidized cyt b (modified from Mojet, 1992).

ently has to work continuously to maintain considerable sodium and potassium gradients.

Sensibly, the sodium pump molecules are localized in the non-rhabdomeric part of the photoreceptor membrane, as shown in blowfly by immunofluorescence and immunogold cytochemistry (Baumann *et al.*, 1994). In addition, the mitochondria tend to concentrate near their customers in the cell boundary (see Fig. 1).

7.2. Flavoproteins and NADH

Redox changes of mitochondrial flavoproteins and NADH are quite exquisitely measured by fluorescence (Scholz *et al.*, 1969) and this approach has been favourably applied to white-eyed fly mutants. The emission spectrum induced by blue (477 nm) light peaks at about 540 nm and its amplitude increases upon hypoxia, characteristic for an increase in reduced flavoprotein (presumably lipoamide dehydrogenase); the ultraviolet (360 nm) induced emission spectrum, peaking at 450 nm, decreases upon hypoxia, signifying an increase in reduced NADH (Tinbergen and Stavenga, 1986). These observations enable the interpretation of dynamic phenomena occurring in a normally functioning retina upon bright illumination.

Illumination of a dark-adapted blowfly mutant *chalky* yields a rapid, biphasic increase of the blue-induced, green emission (Stavenga and Tinbergen, 1983). The fluorescence increase indicates an enhanced oxidation of mitochondrial flavoprotein. Simultaneously, there is a drop in the ultraviolet-induced blue emission, the earmark of an increase in oxidized NADH. Absorbance measurements demonstrate that light adaptation causes a slight absorbance decrease at 432 nm, indicating that cyt b becomes somewhat more oxidized (Fig. 15; Mojet, 1992). The optical changes all point to an enhanced electron flow through the respiratory chain resulting in a light-induced increase in oxygen consumption. Indeed, direct measurements of the oxygen consumption in isolated eye-cups yielded action spectra very similar to sensitivity spectra measured electrophysiologically (Hamdorf and Langer, 1966). Action spectra for both activation and adaptation of the blue, light-induced green fluorescence measured on eyes of intact flies (Fig. 16) agree with the sensitivity spectrum of photoreceptors R1–6 (Tinbergen and Stavenga, 1987), reinforcing the conclusion that activation of the phototransduction process boosts mitochondrial activity, i.e. ATP production.

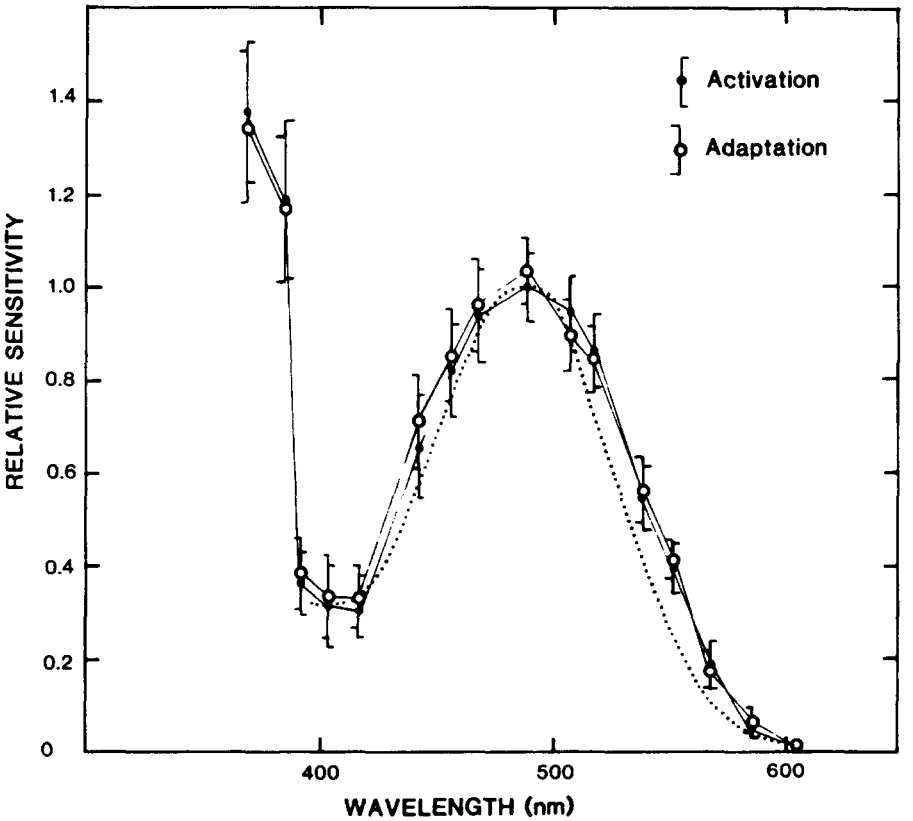


Fig. 16. Spectra for mitochondrial activation and adaptation. For the activation spectrum, a criterion change in blue-induced, green fluorescence caused by a short stimulus flash of variable wavelength was determined. For the adaptation spectrum, a similar criterion was set for a long adaptation light of variable wavelength. The dotted line represents the rhodopsin absorbance spectrum (from Tinbergen and Stavenga, 1987).

7.3. Pumps and Channels

The prime result of phototransduction is the opening of ion channels in the photoreceptor cell membrane. This not only causes influx of calcium, but also a massive influx of sodium as well as an efflux of potassium (Hamdorf *et al.*, 1988; Hardie, 1991a, 1995; Weckström *et al.*, 1991). These light-induced ion currents are counteracted by an enhanced sodium pump activity. Evidently then, phototransduction, sodium pump and mitochondrial activity are intimately coupled. The precise mechanism has yet to be resolved, but there is good evidence that the intracellular calcium concentration plays a crucial role at several levels (Fig. 9). Firstly in the control of a number of primary phototransduction reactions, but also in the control of mitochondrial key enzymes (see Fein and Tsacopoulos, 1988; cf. Hamdorf *et al.*, 1988). The intensity dependence of receptor potential and mitochondrial activity underscores the probable role of calcium in mitochondrial regulation (Fig. 17). Electrophysiological analyses indicate that the depolarization drop from the peak to the plateau value is due to down-regulation of the receptor gain by the increased intracellular calcium concentration (Sandler and Kirschfeld, 1991). The intensity range of mitochondrial activation appears to correspond with the range where peak and plateau deviate.

Because the sodium pump is electrogenic, a hyperpolarizing afterpotential follows the receptor potential induced by a bright flash. From measurements of the afterpotential and the accompanying membrane resistance changes, Jansonius (1993) calculates that the membrane of the blowfly photoreceptor must be very densely packed with sodium pump molecules in order to keep the ion fluxes in

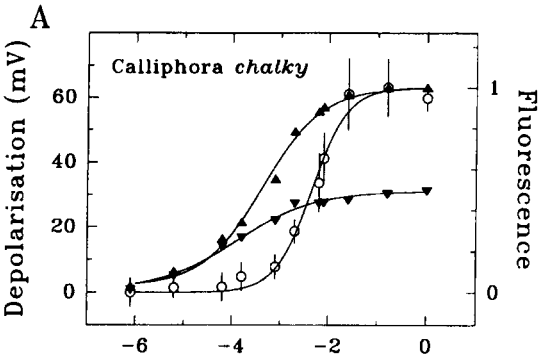


Fig. 17. Intensity dependence of receptor potential peak (upward-pointing triangle) and plateau (downward-pointing triangle) together with the intensity dependence of the mitochondrial activity (open circles), measured through the blue-induced, green fluorescence (from Mojet *et al.*, 1991).

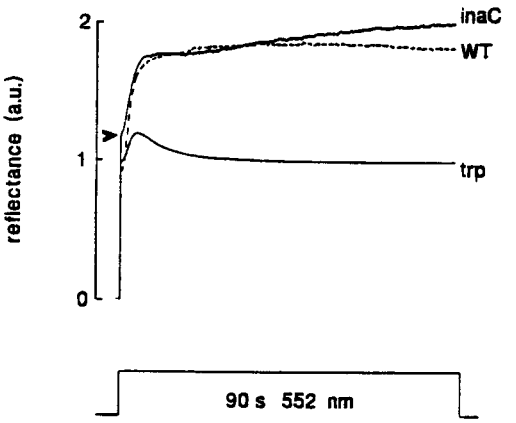


Fig. 18. Reflectance measurements of the pigment migration induced by a step of saturating green (552 nm) light after a preceding dark adaptation time of 2 min. The time course of the reflectance increase in wild-type (WT) indicates a monotonic migration of the photoreceptor pigment granules towards the rhabdomere. In *inaC*, the initial reflectance is distinctly higher than that in WT, demonstrating that the pigment granules were not fully withdrawn from the rhabdomere, thus indicating that calcium is only partly removed from the cell. In *trp*, the step response is transient, indicating that the increase in cellular calcium is only short-lived (from Hofstee and Stavenga, 1995).

hand. A measured electrogenic pump current in the dark of –0.45 nA implies 4.7×10^{-15} mol·s^{-1} ATP hydrolyzed in the dark. With a typical membrane area of the photoreceptor soma of 4×10^3 μm^2 and a maximum density of pump molecules of 10^4 molecules·μm^{-2} possible, yielding a total of 4×10^7 pump molecules per cell, means that each pump molecule hydrolyzes 70 ATP molecules per second in the resting state, rather close to the maximum pump rate of 200 s^{-1} which is realized during bright light (Jansonius, 1993). The visual sense cells hence seem to waste quite some energy in the dark, but recent work by Weckström *et al.* (1991) has illuminated the rationale. Blowfly photoreceptors possess voltage-dependent potassium channels that are slightly leaky at the resting potential, thus necessitating active pumping. These channels are delayed rectifiers, which progressively open upon depolarization and hardly inactivate. The functional reason for this mechanism is that the opening of the potassium channels causes a considerable decrease in membrane resistance, or, in the membrane RC-time. Hence, the extra metabolic energy is spent to improve the photoreceptor's response dynamics (Weckström and Laughlin, 1995).

The fast light response of blowfly photoreceptors is quite different from the slow response of crane-fly (tipulid) photoreceptors, that lack the delayed rectifier, but have instead a rapidly-inactivating potassium channel (Laughlin and Weckström, 1993). The potassium channels of *Drosophila* photoreceptors take a position intermediate between these extremes (Hardie, 1991b). A comparative survey of potassium channels in dipteran photoreceptors revealed the striking fact that the characteristics of the potassium channels are tuned to the animal's flight speed: the high temporal visual resolution of the agile blowflies necessitates the delayed rectifiers, whereas the moderate frequency response of the sluggish crane-flies is served well by the inactivating potassium channels (Weckström and Laughlin, 1995).

8. PUPILLARY PIGMENT

Illumination of fly photoreceptor cells with bright light induces radial migration of pigment granules towards the rhabdomere, thus acting as an intracellular light control or pupil mechanism (Kirschfeld and Franceschini, 1969). The light-induced pigment migration is measurable in intact, living animals as a reduced transmittance and an increased reflectance (Franceschini and Kirschfeld, 1976; Stavenga, 1979). This phenomenon provides a powerful, non-invasive tool for fly photoreceptor research (Fig. 18).

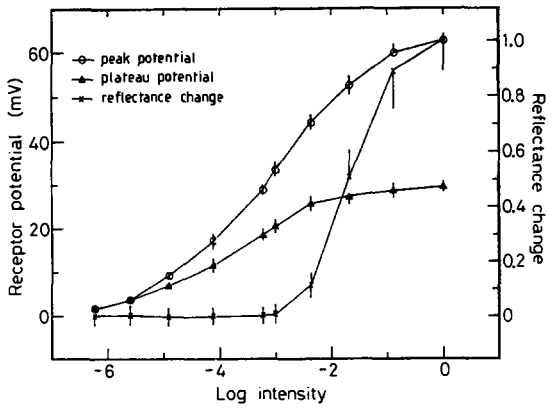


Fig. 19. Intensity dependence of receptor potential peak and plateau together with that of the pupillary reflectance change (from Roebroek and Stavenga, 1990b).

Comparing various eye pigment mutants, Franceschini and Kirschfeld (1976) discovered that the pigment colouring the pupillary granules in *Drosophila* photoreceptor cells belongs to the class of ommochromes (see also Stark and Sapp, 1988). Furthermore, by measuring the eye transmittance in a number of phototransduction mutants, Lo and Pak (1981) demonstrated that no pigment migration occurs in severe *norpA* mutants. Because *norpA* also has no light-induced receptor potential (Section 6), one could be tempted to see this defect as the cause for the absence of pigment migration. However, the receptor potential is certainly not simply the input driving the pupillary motor: when extracellular calcium is diminished by EGTA only the migration of the pupillary pigment granules and not the receptor potential is blocked (Kirschfeld and Vogt, 1980). The *norpA* results prove that receptor potential and pigment migration mechanism have only a partially shared pathway; furthermore, calcium clearly is crucial for driving the pigment granules. This finding immediately suggests that the pigment migration, or rather the resulting change in transmittance and reflectance, can be used as a monitor of the intracellular calcium concentration (Kirschfeld and Vogt, 1980). The functional relationship between calcium and degree of pigment migration, which is probably nonlinear and may be dependent on the mutation, has yet to be determined.

Although the mechanism of the pigment migration is far from being understood in detail, the present evidence points to a central role for microtubules serving as tracks along which the pigment granules are propelled (Wilcox and Franceschini, 1984; Stavenga, 1989). An increase in calcium induces accumulation, i.e. movement of the photoreceptor pigment granules towards the rhabdomere, and a decrease in calcium favours the opposite movement. Presumably, the net direction of the pigment migration is determined by the local calcium concentration (Roebroek and Stavenga, 1990b). The intensity threshold of the pupillary pigment migration corresponds with the peak-plateau transition of the receptor potential (Fig. 19), similar to that of the mitochondrial activation (Fig. 17), adding weight to the hypothesis that intracellular calcium controls the driving force for the pigment granules.

The potential of the pupil mechanism as an endogenous, intracellular calcium probe has so far only been exploited in a small number of *Drosophila* phototransduction mutants. The transient receptor potential mutant, *trp*, has a transient pupillary response (Lo and Pak, 1981), quite similar to the light-induced change in calcium measured photometrically (Peretz *et al.*, 1994a, b); see Fig. 18. This demonstrates that the TRP channel is required for maintenance of the light-induced change in the intracellular calcium concentration.

The pupillary response in the mutant *inaC* is protracted compared to that in wild-type (Hofstee and Stavenga, 1995), similar to the photometric measured time course of calcium in *inaC* (Peretz *et al.*, 1994b). Remarkably, whereas the pupil in wild-type opens in the dark, i.e. the pigment granules are fully withdrawn from the rhabdomere (see Fig. 1), in *inaC* the dark-adapted pupil stays partly closed (Fig. 18; Hofstee and Stavenga, 1995), suggesting that the photoreceptor PKC is vital in removing cellular calcium, either by sequestration or extrusion from the cell (Hardie *et al.*, 1993).

The main function of the pupil mechanism is to control the incident light propagating in the rhabdomere. The light absorbed by the visual pigment in the rhabdomere activates the phototransduction cascade, and this process in turn controls the amplitude of the pigment migration, presumably via calcium. Clearly, phototransduction and pupil mechanism together form a control feedback system, extending the working range of vision (Howard *et al.*, 1987; Juusola *et al.*, 1994), not unlike that of the retinal photoreceptors and the pupil in the human eye.

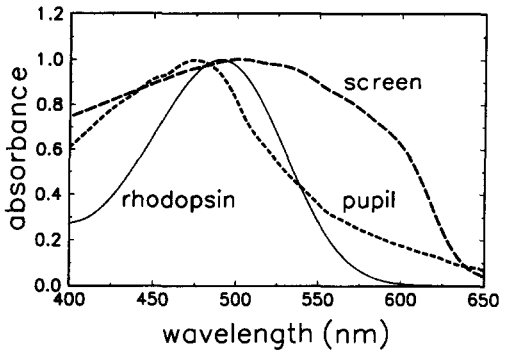


Fig. 20. Absorbance spectra of blowfly screening pigments and pupillary pigment together with the rhodopsin absorbance spectrum demonstrating the red leakage of the pigments, which serves to photoregenerate rhodopsin from metarhodopsin; compare Fig. 2 (after Stavenga *et al.*, 1973).

The positioning of the fly pupil, inside the receptor, may seem a less adequate construction than that in the human eye. However, under bright light the microvilli in the distal section of the photoreceptor, where the pupil is concentrated, are stimulated into saturation, and then they no longer contribute to contrast detection (Roebroek and Stavenga, 1990a).

The visual field of a fly photoreceptor, having an acceptance angle of ca. 1°, can easily embrace the whole sun. Radiating 4×10^{16} absorbable quanta·cm^{-2}·s^{-1} to a fly with photoreceptor absorbance efficiency 0.3 yields that more than 10^{10} photons can be absorbed per second by its visual pigment molecules, i.e. each molecule then absorbs a light quantum at least every 10 ms. Howard *et al.* (1987) determined that a fly photoreceptor saturates when absorbing in excess of 10^9 effective photons per second. The sun light thus is about 1 log unit above saturation. Even from the blue sky, with a 4 log unit lower effective irradiance, the quantum hit per visual pigment molecule is more than once per 100 s, or, 10 visual pigment molecules per microvillus are hit per second. As with the human eye, this would mean that receptor performance becomes limited under natural illumination without protection by the pupil mechanism.

In contrast to the human pupil, which has a flat absorbance spectrum and hence is equivalent to a variable grey filter, the absorbance spectrum of the pupillary pigment in the blowfly peaks in the blue (Fig. 20): the pupil acts as a distinct blue (absorbance) filter (Stavenga *et al.*, 1973). Although a quite impressive reduction of light flux is reached, well over 2 log units at the absorbance peak wavelength (Roebroek and Stavenga, 1990b), the pupil absorbance spectrum seems to be rather badly designed for a light filter in front of a broad-band, blue–green absorbing visual pigment. Again, the filter is less inadequate than it appears. The pupil is driven to closure at rather high intensities, i.e. where the visual pigment rapidly reaches a photosteady state. Without the pupillary filter, natural broad-band light establishes a photosteady state with ca. 30% of the visual pigment molecules in the metarhodopsin state, but a closing pupil distinctly reduces this amount, by at least a factor of 2 (Stavenga *et al.*, 1973; Stavenga, 1980). In this way, the selective, enzymatic degradation of visual pigment molecules is reduced (see Section 3.2). Furthermore, the reduced amount of metarhodopsin molecules means a lower demand for arrestin molecules. The resulting higher amount of unbound arrestins will more rapidly inactivate newly created metarhodopsins.

In addition to controlling the magnitude of light flux in the photoreceptor, the pupil has ancillary functions in improving visual performance. The acceptance angle narrows when the pupil closes, thus increasing spatial acuity upon light adaptation (Hardie, 1979; Smakman *et al.*, 1984) and it shifts the spectral sensitivity of the photoreceptor (Hardie, 1979; Vogt *et al.*, 1982).

9. SCREENING PIGMENTS

The membrane-bounded pigment granules in the primary and secondary pigment cells of the higher flies contain ommochromes, the dominant ocular pigments, specifically xanthommatin. In *Drosophila*, drosopterins predominantly populate the secondary pigment cells (Summers *et al.*, 1982; Stark and Sapp, 1988; Schraermeyer and Dohms, 1993). The pigments are photostable, but their absorbance spectrum distinctly depends on the redox state (Langer, 1975). The general spectral characteristic is a drastic drop of the absorbance in the red, above ca. 600 nm (Goldsmith, 1965; Stavenga *et al.*, 1973; Langer, 1975). Depending on the optical density and the mixture of pigments, the resulting eye colour can rather vary: the eye of the wild-type fruitfly *Drosophila* is cherry-red, whilst that of the housefly *Musca* and the blowflies

Calliphora and *Lucilia* is red–brown; also, the eyes of the hoverflies *Syrphus balteatus* and *Eristalis tenax* are red and virtually black, respectively.

The pigment cells are often called screening pigment cells because their main function is to act as an absorbing screen for off-axis incident light that otherwise would deteriorate visual acuity. High visual acuity requires that light incident from only a narrow spatial angle activates a photoreceptor. Given the construction of a compound eye, blockage of obliquely incident light needs very densely pigmented cells surrounding the visual sense cells. However, the pigment cells of fly eyes fail to meet this criterion adequately in the longer wavelength range. Red light can more or less freely roam through the eye, potentially able to unwantedly activate the visual sense cells. There appears to be a very good rationale for the red-leakiness; the reason is similar to that given for the blue-absorbing pupillary filter. Recall that the blue–green absorbing rhodopsin only marginally absorbs red light, quite in contrast with its photoproduct, metarhodopsin, that strongly absorbs in the red (Fig. 2). Hence, red stray light will preferentially reconvert metarhodopsin back into its original rhodopsin state.

The absorbance spectrum of the pupil pigment peaks in the blue and is low in the orange–red; that of the pigment cells spans a much broader range (Fig. 20). The functional reason for this difference may be that the pigment cells have to meet two conflicting demands: they have to cover the photoreceptor's rhodopsin for obliquely incident light whilst at the same time expose the metarhodopsin. The optimization task of the pupil may be somewhat different: in addition to reducing the metarhodopsin fraction, it seems to be beneficial to increase acuity and shift spectral sensitivity (see Section 8).

The theme of spectral tuning the photostable pigments to the photochemistry of the visual pigment can be elaborated in the light of recent spectroscopical work on genetically-modified fly visual pigment (Britt *et al.*, 1993). Visual pigment research of the last decades has underscored that the chromophore and its protein environment together determine a visual pigment's absorbance spectrum (Stavenga *et al.*, 1991; Hargrave and McDowell, 1992). The position of the absorbance spectrum's peak depends on a number of crucial amino acids, which have been identified by systematic modification of the protein (e.g. Nathans, 1990) and by comparative sequence analysis (Chang *et al.*, 1995). Britt *et al.* (1993) modified different α-helixes of the R1–6 rhodopsin of *Drosophila* and so discovered that the absorbance spectra of the rhodopsin and metarhodopsin state are determined, independent from each other, by different sections of the protein. For a fly in evolution, this means that the precise rhodopsin and metarhodopsin absorbance spectra, i.e. their wavelength positions, can be selected independently from each other.

A photoreceptor's spectral sensitivity, or, its rhodopsin absorbance spectrum, is presumably set for optimal visibility of objects in the environment. In addition, the same selective pressure, i.e. optimal contrast detection (Laughlin, 1994), calls for a screening pigment that absorbs stray light in the wavelength range of the spectral sensitivity. Letting the pigment screen become leaky in the red is rewarded because red stray light can be used for reconversion of existing metarhodopsin molecules, i.e. for photoregeneration of the visual pigment (Stavenga, 1979, 1992).

10. COLOUR AND POLARIZATION VISION

Fly photoreceptor research has been mainly focused on the large visual sense cells, R1–6. From a detailed analysis of the housefly (*Musca*) eye, it appeared that throughout the eye, the R1–6 cells all have the same rhodopsin and therefore form a homogeneous set (Franceschini *et al.*, 1981; Hardie, 1985). The situation for the central cells, R7 and R8, is distinctly more complex, however. Whereas the rhabdomeres of the R1–6 cells are long, independent optical waveguides, traversing the whole retinal layer, the rhabdomeres of R7 and R8 are positioned in tandem, are together as long as an R1–6 rhabdomere, and form together one lightguide. Light that an R7 rhabdomere failed to absorb continues its way in the underlying R8 rhabdomere and thus chances to be absorbed there (Fig. 1).

Two major types of photoreceptors R7 can be distinguished from their pale (R7p) and yellow (R7y) appearance in sections observed with a normal transmission light microscope. R7p has an ultraviolet rhodopsin with a blue metarhodopsin (R340–M460) and its underling, R8p, possesses a blue rhodopsin (R460). The spectral sensitivities of R7p and R8p

closely conform to the absorbance spectra of their visual pigments. The situation for R7y and R8y is quite different, however. R7y has a violet rhodopsin (R430–M510) and its companion R8y a green rhodopsin (R520); (Hardie 1985, 1986; Feiler *et al.*, 1992). Like the visual pigment in the R1–6 cells, that of R7y binds 3-hydroxy-retinol and this also acts as a sensitizer here, thus boosting ultraviolet sensitivity (Hardie, 1985, 1986). Moreover, the rhabdomeric membrane of the housefly R7y photoreceptor houses another photostable pigment, a mixture of the carotenoids lutein and zeaxanthin (Kirschfeld, 1986; Hardie, 1986). These carotenoids may have an important function by quenching oxygen radicals so to protect the photoreceptor from photochemical damage (Kirschfeld, 1982). In any case, they act as a blue absorbance filter, so that ultimately the spectral sensitivity of R7y peaks at 355 nm. The spectral sensitivity of R8y is also much narrowed and peaks at 530 nm (Hardie, 1985, 1986). The central photoreceptors clearly play a crucial role in fly colour vision. Possibly the R7p/R8p and R7y/R8y pairs form a two-colour opponent system (Troje, 1993).

On top of this complex organization, the eyes of male houseflies have a dorsal frontal area where the R7's are blue–green sensitive, identical to R1–6. Similar to these photoreceptors, the R7 receptors in the male dorsal area emit a red fluorescence and therefore are called R7r. The anatomy of the male dorsal area suggests that the signals of R1–6 and R7 are added, apparently in order to improve spotting a potential partner as a contrasting dot against the sky (Hardie *et al.*, 1981). Finally, fly eyes possess a dorsal margin or rim with large photoreceptors (R7marg and R8marg), that are both ultraviolet sensitive, spectrally identical to R7p, as well as strongly polarization sensitive (Hardie, 1984, 1986). Their function is to analyze the polarization pattern of the sky, presumably to use that information in orienting behaviour.

In addition to their large compound eyes, flies possess three small ocelli, each consisting of a set of hundreds of visual sense cells, covered by a single lens. The spectral sensitivity of the ocellar photoreceptors peak in the violet, at 420 nm (*Drosophila*, Hu *et al.*, 1978; *Musca*, Kirschfeld, 1986).

Various rhodopsins have been genetically identified in *Drosophila*. Ectopic expression of the ultraviolet rhodopsins in the R1–6 cells combined with a thorough physiological analysis of these amenable cells (Feiler *et al.*, 1992) demonstrated that the Rh1 gene expresses the main blue–green rhodopsin, R480, of photoreceptors R1–6; the Rh2 gene codes the violet rhodopsin, R420, of the ocelli; the Rh3 and Rh4 genes code two distinct ultraviolet rhodopsins, R345 and R375, respectively, expressed in nonoverlapping sets of R7 cells (Montell *et al.*, 1987; Pollock and Benzer, 1988; Carulli *et al.*, 1994). The resulting spectral sensitivities resemble those of the *Musca* R7p and R7y cells, respectively. Rh3 is also expressed in the R7marg and R8marg cells (Fortini and Rubin, 1990). The gene for the R8 opsins has yet to be identified. Although the organization of the photoreceptors and their visual, sensitizing and carotenoid pigments appears to be rather similar among the different flies, certain subtle variations exist, e.g. the UV-absorbing sensitizing pigment seems to be absent in *Drosophila* 7y cells (see Feiler *et al.*, 1992).

11. PIGMENTS OF INSECT EYES

The wealth of information gathered on the fly visual system forms a solid basis for further insight into other insect eyes. The anatomy and optics of their eyes, the photochemistry of the visual pigments, the phototransduction process, the pupil mechanism and the pigments in the pigment cells of virtually any insect conform to rather similar rules. Nevertheless, the common themes allow many variations. These will be touched in this, final part.

11.1. Visual Pigments and Chromophores

In both vertebrate and invertebrate opsins, the chromophore is invariably bound to the ε-amino group of a lysine in helix 7 of the seven-helical transmembrane spanning opsin. The absorbance spectrum crucially depends on the interaction of the chromophore with specific amino acids in the opsin (Hargrave and McDowell, 1992; Chang *et al.*, 1995). Curiously, the shape of the main absorbance band remains identical when plotted as a function of log-wavelength (MacNichol, 1986; Stavenga *et al.*, 1993).

The chromophore of insect visual pigments is either retinal (e.g. bees and locusts) or 3-hydroxy-retinal (e.g. butterflies and flies); a sensitizing

pigment, boosting sensitivity in the ultraviolet, is found so far only among flies (Vogt, 1989).

Presumably, the photochemical cycle does not depend on the chromophore, as rhodopsin conversion always ends in a thermostable (and reconvertible) metarhodopsin state. However, few further details are known of the photochemical cycle of insect visual pigments in general. A number of intermediate states of only the ultraviolet rhodopsin of the neuropteran *Ascalaphus* have been characterized (Hamdorf, 1979). From the present knowledge gained on the fly and comparing that to similar results from extensive studies on vertebrate rhodopsins, it may be conjectured that the thermal decay of photoexcited rhodopsin goes through a rather standardized series of intermediates, similar to that of the fly (Stavenga *et al.*, 1991), leading to exposition of the binding site for the photoreceptor G-protein.

Metarhodopsin is selectively degraded at high speed in butterfly eyes (Bernard, 1982; Bernard and Remington, 1991). In nymphalids, the process is first-order with time constant 77 min at 12.5°C, 18 min at 23°C and 4 min at 26.5°C (Bernard, 1982). The recovery of butterfly rhodopsins is kinetically complex and presumably depends on the reservoir of the components for the rhodopsin production line: at 26.5°C a time constant of 6 min was measured for small conversions, but this value went up to 111 min for large conversions (Bernard, 1982). A photoisomerase that converts all-*trans*-retinal to 11-*cis*-retinal was isolated from bees (Schwemer *et al.*, 1984) and its principal localization was identified as the primary pigment cells; in the bee retinol is also formed (Smith and Goldsmith, 1991). The results correspond with earlier findings in the fly (Schwemer, 1989, 1993; Section 3.2).

11.2. Phototransduction and Respiration

The light-induced receptor potentials measured in insect photoreceptors have a very similar shape, although the time course can rather differ. The response to a light step always is a graded depolarizing response that peaks after several ms and then falls back to a plateau. As described above, numerous control processes are already active at the first levels of phototransduction, with probably the intracellular calcium concentration and the voltage-dependent potassium channels as the dominant factors shaping the receptor response. The regulation of intracellular calcium may vary somewhat among insects, however (see Sandler and Kirschfeld, 1992).

Recognition of the importance of oxidative metabolism for the efficient functioning of insect photoreceptors came from the early studies of Autrum and Tscharntke (1962) and Hamdorf and Langer (1966). They measured the oxygen consumption of eye-cups with sensitive microrespirometers and showed that the oxygen consumption was considerably enhanced by light, especially in the diurnal flies. Measurements on the large dorsal eye of the drone bee with oxygen-sensitive microelectrodes have further advanced our knowledge of photoreceptor respiration. Like in the fly, the action spectrum of the light-induced oxygen consumption by the drone bee eye is in good agreement with the absorbance spectrum of the main rhodopsin (R446). Excessive conversion of the violet rhodopsin to its blue–green metarhodopsin, M505, yields a prolonged afterpotential as well as extended oxygen consumption (Jones and Tsacopoulos, 1987). Furthermore, the intensity range of light-induced respiration is shifted upwards over a few log units with respect to that of the late receptor potential (Tsacopoulos and Poitry, 1982). Most likely, the mechanism for mitochondrial activation is similar among insect photoreceptors.

11.3. Light Control Systems

Virtually all diurnal insects investigated have vigorous pupil mechanisms inside their photoreceptor cells. Yet, the speed of closure and opening, as well as the span of intensity control is unsurpassed in the higher flies. The time constant of the pupil in flies is of the order of seconds, but in other insects, the process is often distinctly slower, i.e. occurring over several tens of seconds, in bees, or even minutes, in crickets (Stavenga, 1979, 1989).

Migration of the pigment granules in the pigment cells, or even movement of complete cells, is also widespread among insect eyes. However, this type of pigment migration is usually coupled to a diurnal rhythm. The biological function is to widen the angular sensitivity thereby increasing absolute light sensitivity when photons are scarce. Quite extreme examples of pigment migration are encountered in the eyes of moths and certain orthopterans (Rossel, 1979; Stavenga, 1979). The strong dependence on the

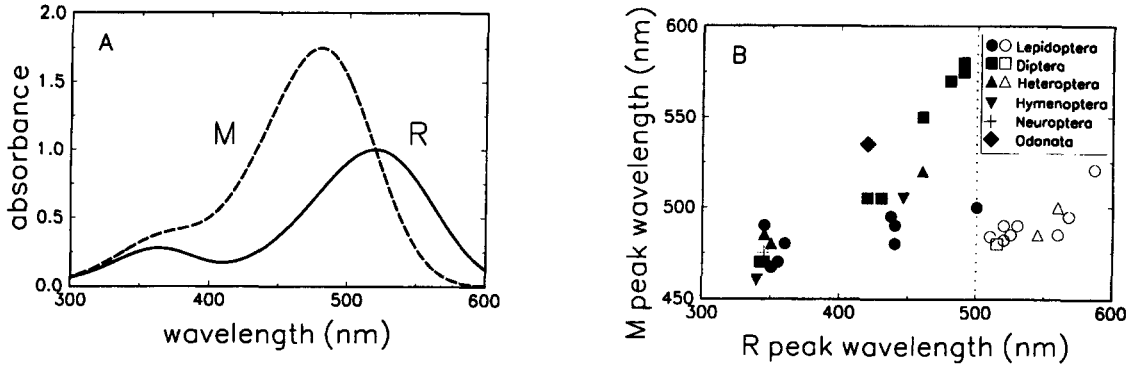


Fig. 21. (A) Absorbance spectra of a green rhodopsin and its blue absorbing metarhodopsin. (B) Peak wavelength of rhodopsin versus that of metarhodopsin (modified from Stavenga, 1992).

species is exemplified by Ro and Nilsson (1993, 1995). In the so-called superposition eyes, usually found in nocturnal insects, substantial cellular and pigment movements occur, that are sometimes not triggered by the visual sense cells, but by photosensitive structures near the lenses (Nilsson *et al.*, 1992). From a comparison of the pupillary screening-pigment migration in the superposition eyes of a noctuid moth and a dung beetle, Warrant and McIntyre (1995) conclude that the extent of pigment migration and its effect on light flux and visual acuity is intimately coupled to the range of light intensities that the animals experience during their active period, i.e. the action of the pupil mechanism is determined by the animal's visual ecology.

11.4. Visual Ecology

The optical organization of the fly retina is rather extraordinary when compared with other insects. Usually the rhabdomeres, i.e. the parts of the photoreceptors containing the visual pigments, of an ommatidium are fused together, so forming together one optical waveguide, the case in the so-called apposition eyes of bees and butterflies; a comparable situation exists in the optical superposition eyes of moths (Nilsson, 1989).

Commonly insect eyes possess three types of photoreceptor cells with different visual pigments, thus providing the basis for colour vision. Except for the bee (Menzel and Backhaus, 1989), extensive evidence for colour vision is lacking (see e.g. butterflies: Bernard and Remington, 1991). The packing of different visual pigments into one optical waveguide affects the spectral sensitivity of the individual photoreceptors by mutual filtering. Presumably, colour discrimination is optimized in fused rhabdoms.

The tuning of a photoreceptor's spectral sensitivity to the spectral characteristics of its visual field belongs to the realm of visual ecology. Discrimination of objects, e.g. a potential partner or prey, against the ultraviolet/blue sky is best achieved by equipping a visual sense cell with a rhodopsin maximally absorbing in the short wavelength range (Stavenga, 1992). Similarly, discrimination of the patterns of polarization in the sky requires an (ultra-)violet rhodopsin, a common characteristic of insect dorsal rims (Labhart *et al.*, 1984; Rossel and Wehner, 1986; Rossel, 1989). For discrimination of flowers against green plants a green rhodopsin is quite suitable. An interesting class of green visual pigments is that of fireflies. They appear to be finely tuned to the bioluminescence of the animals' lanterns (Lall *et al.*, 1982).

Extending the tuning view to a higher level leads to the question how in general screening pigments will be spectrally tuned to the visual pigments. Parallel cases of the long-wavelength leaky screening pigments of fly eyes are found in only a limited number of insect eyes; e.g. a red-leaky screening pigment exists in the dorsal eye of the drone bee, and a yellow-and-red transparent pigment coexists with the ultraviolet rhodopsin of certain mayflies (Stavenga, 1992). The sparsity of leaky screening pigment is immediately understood from an inventory of the absorbance spectra of known rhodopsin–metarhodopsin pairs. The experimental fact is that

green rhodopsins invariably have a metarhodopsin that maximally absorbs at a shorter wavelength than the peak wavelength of its rhodopsin (Fig. 21A). Only rhodopsins with peak wavelength below ca. 500 nm have a metarhodopsin partner with peak absorbance at longer wavelengths (Fig. 21B). Only eyes with exclusively members of this class of rhodopsin can afford to have long-wavelength leaky screening pigments. Photoreceptors with a green rhodopsin must be shielded with an extended, broad-band grey filter. Because green rhodopsins are most generally encountered in insect eyes, this is indeed the common situation realized in insect eyes (Langer, 1975; Stavenga, 1992).

Optimization at another level of photoreceptor performance occurs with the expression of ion channels that have characteristics tuned to an animal's behaviour (Section 7.3). A most striking example is the day-and-night active locust. Locusts have a strong diurnal rhythm in their retina as is clear from both anatomical and physiological studies. For instance, because of a massive breakdown of visual membrane at dawn and its renewal in the evening, the rhabdomere (and consequently the photoreceptor acceptance angle) is in the daytime much narrower than at night (Williams, 1982, 1983). In addition, the locust photoreceptor's potassium channels are modulated on a diurnal basis: during the day, the dominant conduction is a delayed rectifier potassium channel like that of the blowfly, and at night, it swaps places with a rapidly-inactivating channel, like that of crepuscular dipterans (Cuttle *et al.*, 1995; Weckström and Laughlin, 1995). The result of all diurnal modifications is that the photoreceptor has a high acuity and low sensitivity in the day and a low acuity and high sensitivity at night.

Another exemplary case, demonstrating that all possible optical, photochemical and electrical tricks are geared to achieving the optimal result in life, namely reproduction, is the dorsal eye of the drone bee. The drone's dorsal eye is very large, occupying the major part of the head; the facet lenses are big, so as to capture as much light as possible, whilst securing fine acuity; the visual pigment absorbs violet light, so as to detect a passing queen with maximum contrast; the metarhodopsin absorbs in the blue–green and the screening pigment is red transparent in order to photoregenerate the visual pigment (Menzel *et al.*, 1991; Stavenga, 1992); the photoreceptor membrane harbours a special voltage-dependent Na^+ channel that, together with a counter-balancing K^+ conductance, amplifies changes in the detected light signal (Coles and Schneider-Picard, 1989; Vallet and Coles, 1993; Weckström and Laughlin, 1995).

Finally, we have to note that the ocular pigments of insect eyes not only provide the animal its visual capacities, but that they also determine its visibility. Generally, therefore, the colour of an insect's eye blends in with its body colour, thus improving camouflage or display (Stavenga, 1979).

12. CONCLUSION

The role retinal pigments play in the functioning of insect eyes is gradually beginning to be understood. Clearly, the components of the visual machinery of the retina are often strongly intertwined and finely tuned to each other. The factors setting the criteria of the interplay ultimately must be sought in the animal's behaviour within the constraints set by the environmental elements. The challenge for the near future is to combine the present, rather qualitative knowledge into a more quantitative framework that will not only solidify existing concepts, but also will serve to reveal new design strategies in the visual ecology of insects.

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